

Computer-Guided Approach to Access the Anti-influenza Activity of Licorice Constituents

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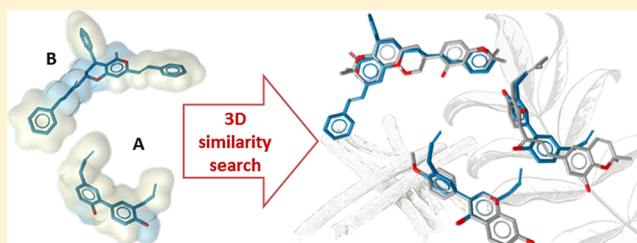
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ABSTRACT: Neuraminidase (NA), a key enzyme in viral replication, is the first-line drug target to combat influenza. On the basis of a shape-focused virtual screening, the roots of *Glycyrrhiza glabra* (licorice) were identified as plant species with an accumulation of constituents that show 3D similarities to known influenza NA inhibitors (NAIs). Phytochemical investigation revealed 12 constituents identified as (*E*)-1-[2,4-dihydroxy-3-(3-methyl-2-butenyl)phenyl]-3-(8-hydroxy-2,2-dimethyl-2H-1-benzopyran-6-yl)-2-propen-1-one (**1**), 3,4-dihydro-8,8-dimethyl-2H,8H-benzo[1,2-*b*:3,4-*b'*]dipyrans-3-ol (**2**), biochanin B (**3**), glabrol (**4**), glabrone (**5**), hispaglabridin B (**6**), licoflavone B (**7**), licorice glycoside B (**8**), licorice glycoside E (**9**), liquiritigenin (**10**), liquiritin (**11**), and prunin (**12**). Eleven of these constituents showed significant influenza virus NA inhibition in a chemiluminescence (CL)-based assay. Additional tests, including (i) a cell-based cytopathic effect inhibition assay (general antiviral activity), (ii) the evaluation of cytotoxicity, (iii) the inhibition of the NA of *Clostridium perfringens* (CL- and fluorescence (FL)-based assay), and (iv) the determination of self-fluorescence and quenching, provided further perspective on their anti-influenza virus potential, revealing possible assay interference problems and false-positive results. Compounds **1**, **3**, **5**, and **6** showed antiviral activity, most likely caused by the inhibition of NA. Of these, compounds **1**, **3**, and **6** were highly ranked in shape-focused virtual screening.



Influenza is an acute viral infection of the upper and lower respiratory tract. In humans, this disease is caused by influenza virus types A (e.g., H3N2 and H1N1) and B. High-risk patients, such as infants, the elderly, and individuals suffering from chronic medical conditions (e.g., heart or lung diseases) or with a weak immune system, are prone to develop severe complications such as pneumonia, which can eventually lead to death.¹ To fight this serious public health threat, two main classes of drugs are available (i.e., M2 ion channel blockers and neuraminidase inhibitors, NAIs). The application of M2 ion channel blockers is limited to influenza A viruses. Moreover, currently circulating influenza virus subtypes H1N1 and H3N2 as well as avian H5N1 influenza viruses are resistant to this class of drugs.^{2–4} Hence, the viral neuraminidase (NA; also known as sialidase) represents the only sensitive, currently established anti-influenza drug target. Influenza virus NA is located on the viral surface, where it catalyzes, for example, the hydrolysis of terminal sialic acid residues from newly built virions.⁵ The enzyme forms a tetramer consisting of four

identical subunits, and only in this assembly state the viral neuraminidase is active.⁶

By application of influenza virus NAIs, the function of the enzyme is blocked, thus halting viral reproduction and spread. To date, NAIs including oseltamivir, zanamivir, peramivir, and laninamivir represent primary treatment options for influenza infections.^{7–9} Until recently, NAI-resistant viruses were detected only sporadically.¹⁰ However, the influenza season of 2007/2008 showed that virulent NAI-resistant strains can be spread worldwide.^{11,12} These developments and the threat of pandemics have raised concerns about the efficacy of the available anti-influenza drugs.

In recent years, many publications have reported the successful targeting of NA by compounds isolated from natural sources.^{13,14} In order to search for new strategies to develop innovative anti-influenza drugs, attention has been given to the

Special Issue: Special Issue in Honor of Otto Sticher

Received: October 1, 2013

Published: December 6, 2013

flexible regions of the 150- and 430-loops.^{15–17} These regions have been shown to potentially cause a widening of the active site, making it accessible to novel inhibitors of distinct molecular shape.^{13,15,18–20}

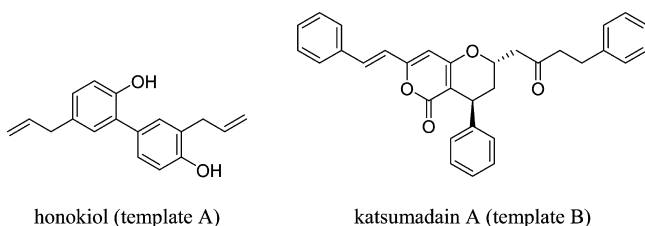
In the current study, using a computational approach, the roots of *Glycyrrhiza glabra* L. (Fabaceae) were identified as a plant source containing constituents that share structural commonalities with previously identified NAIs from other natural sources.^{13,18} Interestingly, in accordance with the computational predictions, the most prominent natural product scaffolds possessing NA inhibitory activity have been confirmed as flavonoids.¹⁴ However, recently it has been suggested that some of these substituted phenyl-benzopyran scaffolds could be problematic in fluorescence (FL)-based NA inhibition assays due to signal quenching, resulting in false-positive results.^{21,22} Hence, in addition to the phytochemical and in-depth biological investigation of licorice constituents, in this report some of the pitfalls of NA-based assays are discussed.

RESULTS AND DISCUSSION

Template Selection for Virtual 3D Similarity Search.

On the basis of experimental data from in-house screening and from the literature, two natural compounds, the neolignan honokiol and the diarylheptanoid katsumadain A (Chart 1), were selected as templates for a similarity search.

Chart 1. Chemical Structures of Two Selected Template Compounds for a 3D Similarity Search



Honokiol is a moderately active inhibitor with an IC_{50} of 3.01 μM against the NA of the historic influenza A strain PR/8/34, as determined in a chemiluminescence (CL)-based NA inhibition assay. Interestingly, its activity is more potent against the oseltamivir-resistant seasonal H1N1 strain B/55/08 (IC_{50} 1.39 μM).

Katsumadain A was discovered as an NAI with an IC_{50} of 1.05 μM (PR/8/34) in an earlier study.¹⁸ With its T-shaped structure (Chart 1), this bulky compound represents an unusual and novel influenza NA inhibiting scaffold. Molecular dynamics simulations and docking have suggested that katsumadain A is likely to bind to an extended (i.e., more widely open) NA binding pocket, a result of the conformational flexibility of the 430- and 245-loops.¹⁸ In a follow-up study, katsumadain A served as a lead structure in finding further highly active and resistance-breaking NAIs using shape-focused virtual screening.¹³

In the present work, honokiol and katsumadain A were used as chemically diverse templates for the identification of plant material with an accumulation of constituents that are likely to be active against influenza NA.

3D Similarity Screening of the TCM Database@Taiwan. To find novel resistance-breaking NAIs from natural sources, the two templates selected were applied to a 3D similarity screening of the TCM Database@Taiwan²³ using the program ROCS. The similarity was quantified using the TanimotoCombo score, which is a combination of shape similarity (ShapeTanimoto score) and chemical similarity (ColorTanimoto score) (<http://www.eyesopen.com/docs/rocs/current/html/index.html>). The TanimotoCombo ranges from 0 to 2. It is the sum of the ShapeTanimoto and ColorTanimoto score, which both range from 0 to 1 and equally contribute to the combined score. The higher the scores, the more similar is the conformation of the database molecule to the conformation of the template. The rank-ordered list of molecules obtained was analyzed regarding their origin, knowledge of anti-influenza/antiviral activity of the TCM plants, and hints from traditional medicine about

Chart 2. Chemical Structures of Licorice Constituents

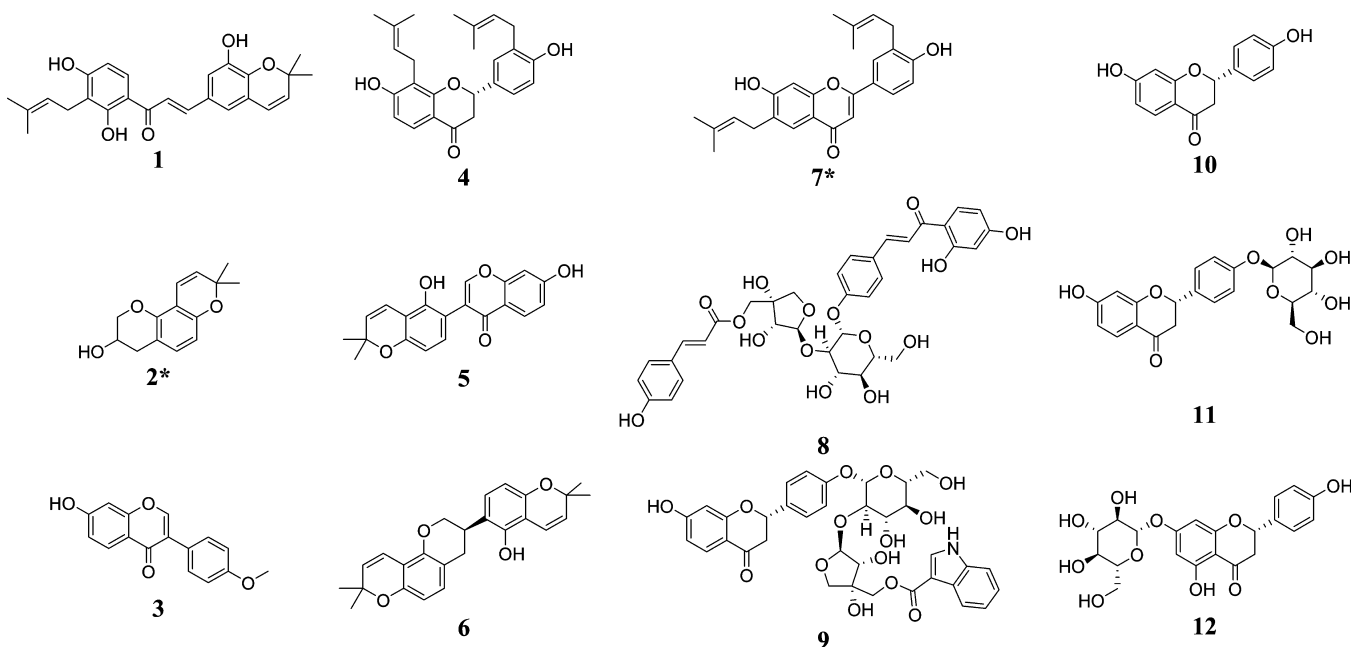
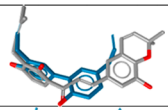
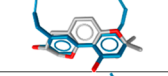
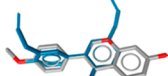
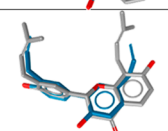
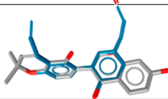
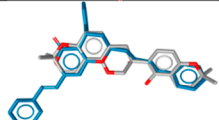
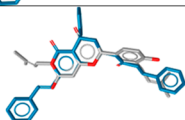
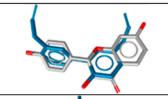
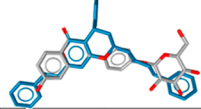
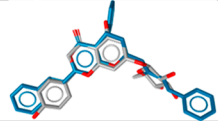


Table 1. ROCS Alignment Data of Compounds Isolated from *G. glabra*

code	most similar template	ROCS alignment with template ^a	Tanimoto combo	shape Tanimoto	color Tanimoto
1	A		0.83	0.56	0.27
2 ^b	A		0.90	0.57	0.33
3	A		0.95	0.62	0.33
4	A		1.07	0.72	0.35
5	A		0.65	0.46	0.19
6	B		0.74	0.53	0.21
7 ^b	B		0.68	0.49	0.19
8 ^b	B	^c	0.55	0.31	0.24
9 ^b	B	^c	0.65	0.55	0.10
10	A		0.97	0.67	0.30
11	B		0.57	0.48	0.09
12	B		0.70	0.55	0.15

^aCarbon atoms of the template structure blue, of the query structure gray. Oxygen atoms red. ^bNot in TCM Database@Taiwan. ^cSimilarity threshold too low.

beneficial effects of natural-based influenza remedies that are known to contain the respective compounds as prominent constituent and accessibility of the plant material.

Selection of Plant Material: *Glycyrrhiza glabra*. Virtual screening indicated that the roots of *Glycyrrhiza* species contain several known constituents (as reported in the TCM Database@Taiwan²³) with significant structural similarities to template A or B.

Considering the degree of similarity between all 103 *Glycyrrhizae radix* database entries and both templates, it was found that they generally show a higher degree of similarity to honokiol than katsumadain A. With a TanimotoCombo score of 0.70 or higher, constituents were identified that have been isolated reportedly from this plant material (i.e., compounds 1, 3, 4, 6, and 10; Chart 2). This prediction prompted the investigation of the roots of *G. glabra* phytochemically and the evaluation of the in vitro NA inhibiting activity of its

constituents, with a preference for compounds similar to the template structures.

Dried and processed roots of *G. glabra* (*Liquiritiae radix*) are commonly referred to as licorice. They are among the oldest and most popular traditional herbal medicines worldwide. For instance, in mainland China, the remedy is known under the name *gan-cao*, which includes the dried roots and stolons of three *Glycyrrhiza* species, i.e., *G. uralensis*, *G. glabra*, and *G. inflata*.²⁴ Major compound classes responsible for the bioactivity of *gan-cao* are triterpene saponins, flavonoids (including isoflavones), and coumarins.^{24–28} As recently reviewed, in particular flavonoids show a wide variety of biological properties, such as antiulcer, antioxidant, anti-inflammatory, antimicrobial, antispasmodic, antitumor, and metabolic syndrome preventive effects.²⁴ Strikingly, the roots of *Glycyrrhiza* species are the most frequently used TCM herbs for treating respiratory tract infections.²⁹ In addition, they are

Table 2. Inhibition of NA of Influenza Viruses A PR/8/34, J/8178/09, B/55/08, and HK/68 by Compounds Isolated from *G. glabra* as Determined in CL-Based Enzyme Inhibition Assay

code	50% NA inhibitory concentration [μ M] against influenza virus A ^a			
	PR/8/34	J/8178/09	B/55/08	HK/68
1	0.25 \pm 0.00	n.d. ^b	1.31 \pm 0.00	n.d. ^b
2	6.47 \pm 2.59	15.5 \pm 5.02	30.6 \pm 35.2	12.4 \pm 4.14
3	1.37 \pm 0.22	3.04 \pm 0.88	5.04 \pm 2.37	5.04 \pm 1.15
4	0.51 \pm 0.00	3.00 \pm 0.97	14.1 \pm 18.3	3.06 \pm 1.21
5	0.40 \pm 0.17	3.22 \pm 1.11	9.42 \pm 0.42	5.34 \pm 2.29
6	1.11 \pm 0.53	2.31 \pm 0.23	1.20 \pm 0.30	2.85 \pm 1.58
7	2.82 \pm 0.36	14.7 \pm 3.71	n.d. ^b	9.18 \pm 3.73
8	0.04 \pm 0.02	n.d. ^b	2.83 \pm 1.12	n.d. ^b
9	0.34 \pm 0.17	n.d. ^b	4.52 \pm 2.34	n.d. ^b
10	2.07 \pm 0.37	n.d. ^b	8.33 \pm 3.03	n.d. ^b
11	83.1 \pm 17.8	n.d. ^b	140 \pm 12.8	n.d. ^b
12	3.00 \pm 0.46	n.d. ^b	4.53 \pm 1.85	n.d. ^b
oseltamivir	0.0003 \pm 0.0001	0.0005 \pm 0.0002	0.08 \pm 0.02	0.0002 \pm 0.0001

^a*n* = 3, each concentration in duplicate. ^bNot determined.

described as antiviral herbal remedies³⁰ against chronic hepatitis B and C, HIV, cytomegalovirus, and Herpes simplex and as ingredients of complex Chinese antiviral preparations such as *ma-xing-shi-gan-tang*,³¹ *feng-refan-wei*,²⁹ and *redu-xi-fei*.²⁹ *Glycyrrhiza* roots are usually applied as honey-roasted licorice, i.e., *zhi-gan-cao*.³²

Identification of Pure Compounds. As a starting point for phytochemical investigation, a methanol extract of licorice was prepared to obtain a multicomponent mixture embracing a wide range of secondary metabolites. Since glycosidic molecules showed only low similarity to both templates, and in order to mimic the use in traditional preparations, extraction in sulfuric acid was chosen with the intention to break down these glycosides. In order to separate the aglycones from the respective sugars, dichloromethane was used to obtain an aglycone-enriched fraction.

Both the methanol extract and the aglycone-enriched fraction were investigated for their NA-inhibiting potential in a CL-based enzyme inhibition assay against H1N1 strain PR/8/34. As a positive control, the well-established NAI oseltamivir (IC₅₀ 0.29 nM) was used. In this assay, the methanol extract showed an IC₅₀ of 1.70 μ g/mL, whereas the aglycone-enriched fraction exhibited slightly more potent NA inhibitory activity, with an IC₅₀ value of 0.33 μ g/mL.

On the basis of these results, both crude preparations were subjected to phytochemical workup. After several chromatographic separation steps, 12 constituents were isolated (Chart 2), namely, (*E*)-1-[2,4-dihydroxy-3-(3-methyl-2-butenyl)-phenyl]-3-(8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-yl)-2-propen-1-one (**1**), 3,4-dihydro-8,8-dimethyl-2*H*,8*H*-benzo[1,2-*b*:3,4-*b'*]dipyrans-3-ol (**2**), biochanin B (**3**), glabrol (**4**), glabrone (**5**), hispaglabridin B (**6**), licoflavone B (**7**), licorice glycoside B (**8**), licorice glycoside E (**9**), liquiritigenin (**10**), liquiritin (**11**), and prunin (**12**). The constituents were identified by using HPLC and comparison of their specific rotations and spectroscopic data with published values.^{33–41}

Compounds **1** to **7** were isolated from the aglycone-enriched fraction, while compounds **8** to **12** were isolated from the methanol extract. All 12 isolates have been reported previously from the roots of *G. glabra*. The isolated *Glycyrrhiza* constituents can be classified as two benzodipyrans (**2**, **6**) and glycosidic (**8**, **9**, **11**, **12**) and nonglycosidic (**1**, **3**–**5**, **7**, **10**) flavonoids.

Compounds **3** and **11** are known from earlier work to exhibit weak activity against the NA of influenza viruses A/PR/8/34 (H1N1), A/Jinan/15/90 (H3N2), and B/Jiangsu/10/2003 in FL-based NA inhibition assays.⁴² Compounds **3**, **5**, **10**, and **11** have been mentioned in Chinese or Korean patents as ingredients of multicomponent medicinal compositions for the treatment of viral respiratory tract infections.^{43–45} However, the anti-influenza actions or detailed studies of these four compounds have not been reported to date.

ROCS Alignment. An overview of compounds identified from *G. glabra* and their similarities to templates A and B is provided in Table 1. Four out of the 12 structures disclosed after their isolation and identification (**2**, **7**, **8**, and **9**) have not been covered by shape-focused screening, since they were not present in the virtual screening library. For the 3D alignments performed with all 12 isolates from licorice, seven molecules identified in the plant material (**1**, **2**, **3**, **4**, **6**, **10**, and **12**) had a reasonably high degree of similarity with the template molecules (TanimotoCombo score ≥ 0.70). The shape similarity was generally higher than the chemical similarity. The latter is a measure of the geometric fit of pharmacophoric features including hydrogen-bond donors/acceptors and hydrophobic, anionic, cationic, and cyclic moieties. The low ColorTanimoto scores can be explained by the fact that this global descriptor (as ShapeTanimoto) does not discriminate between different parts of a molecular scaffold, such as parts representing the pharmacophore and areas of the ligand not crucial for bioactivity. This may lead to an underestimation of how well a pharmacophore is represented in a molecule. This frequently occurs, as exemplified in the case of glycosylated molecules such as compound **12**, where the presence of multiple hydrogen-bond donors in the sugar moiety may drastically lower the similarity score measured against an aglycone, even if the ligand parts responsible for bioactivity are closely related.

Biological Investigations. To evaluate experimentally the inhibitory activity against influenza virus NA, a CL-based enzyme inhibition assay was performed (Table 2). Tests were conducted with a historic H1N1 strain (PR/8/34), an oseltamivir-resistant seasonal H1N1 strain (B/55/08), a virus strain of the pandemic H1N1 lineage that emerged in 2009 (J/8178/09), and a H3N2 virus (HK/68). The resistance of oseltamivir against N1 of B/55/08 was shown previously.^{13,18}

Except for compound **11**, which showed an IC_{50} of 83.1 μM , all compounds studied inhibited the oseltamivir-susceptible viral NA of influenza virus PR/8/34 at concentrations lower than 10 μM . In general, the NA of the oseltamivir-resistant B/55/08 was less susceptible to most of the compounds. However, similar IC_{50} ranges were determined for **6** and **12** against oseltamivir-susceptible and -resistant H1N1 strains.

To further investigate the anti-influenza potential of the constituents from *G. glabra*, cytopathic effect (CPE) inhibitory assays were performed with the pandemic H1N1 J/8178/09 and the H3N2 HK/68 influenza A virus (Table 3). To reduce

Table 3. Cytotoxicity and Inhibition of the Cytopathic Effect (CPE) by Compounds Isolated from *G. glabra* as Determined in MDCK Cells

code	50% CPE inhibitory concentration [μM] ^a		50% cytotoxic concentration [μM] ^b
	J/8178/09	HK/68	
1	29.7% ^c	48.1% ^c	135 \pm 36.3
2	36.1% ^c	n.a. ^d	336 \pm 38.4
3	38.2 \pm 4.79	42.6 \pm 0.49	123 \pm 48.3
4	n.a. ^d	n.a. ^d	25.4 \pm 2.58
5	34.7 \pm 14.8	24.2% ^c	90.8 \pm 34.9
6	48.4% ^c	31.2% ^c	39.2 \pm 15.9
7	34.2% ^c	n.a. ^d	79.7 \pm 29.9
8	n.a. ^d	n.a. ^d	>144
9	n.a. ^d	n.a. ^d	>144
10	n.a. ^d	n.a. ^d	301 \pm 25.5
11	n.a. ^d	n.a. ^d	>239
12	49.6% ^c	n.a. ^d	126 \pm 34.5
oseltamivir	0.03 \pm 0.01	0.004 \pm 0.002	n.d. ^e

^a $n = 2$ to 3, each concentration once. ^b $n = 3$, each concentration in triplicate. ^cPercentage of maximal inhibition of the CPE at a concentration of 50 μM . ^dNot active (up to 50 μM). ^eNot determined.

the risk of nonspecific compound action, cytotoxicity was determined in parallel (Table 3). Most compounds were well tolerated by MDCK cells. Compound **12** did inhibit the CPE of J/8178/09 by about 50%. The others, including **8–11**, showed no effect. In contrast, and with the exception of **4**, all compounds obtained from the aglycone-enriched fraction exhibited antiviral activity against at least one of the viruses studied.

Alternative Enzyme Inhibition Assays Using the Bacterial NA of *Clostridium perfringens*. Recently, concerns were raised about the reliability of NA inhibition assays for the testing of natural compounds, in particular for flavonoids in combination with FL-based assays.^{21,22} FL quenching was shown to lead to false-positive results with natural compounds containing a substituted phenyl-benzopyran scaffold.

It was investigated as to whether licorice-derived compounds can lead to such unfavorable effects by using an FL-based assay. However, this assay did not work for viral NA as tested for compound **3** (results not shown), a compound that inhibited J/8178/09 and HK/68 in the CPE inhibition assay. In contrast, the FL-based assay works with the NA from *C. perfringens*. This allowed a comparative study of compounds **2** to **7** isolated from the aglycone-enriched fraction with this bacterial NA in an FL- as well as CL-based assay (Table 4). The amounts isolated of compound **1** were not sufficient for further testing.

Table 4. Self-Fluorescence, Quenching, and Inhibition of the NA of *C. perfringens* by Compounds Isolated from *G. glabra* as Determined in FL- and CL-Based Enzyme Inhibition Assay

code	self-fluorescence [%] at 100 μM	quenching [%] at 100 μM	50% NA inhibitory concentration [μM]	
			FL assay	CL assay
2 ^a	0.00	15.0	2.88 \pm 2.23	0.64 \pm 0.63
3 ^a	15.4	−12.4	13.6 \pm 8.13	0.34 \pm 0.22
4 ^a	1.18	37.4	2.17 \pm 0.64	0.13 \pm 0.08
5 ^a	0.68	28.3	11.7 \pm 6.62	0.07 \pm 0.04
6 ^a	0.05	15.1	4.23 \pm 2.27	0.17 \pm 0.04
7 ^a	3.98	7.10	4.23 \pm 1.57	0.35 \pm 0.09
oseltamivir ^b	0.05	12.8	125	110

^a $n = 2$, each concentration in duplicate. ^b $n = 1$.

Self-fluorescence and quenching effects were evaluated at 100 μM . The percentage of self-fluorescence was calculated setting the fluorescence of 4-methylumbelliferone (4-MU; cleavage product) in the test buffer to 100%. To determine possible quenching effects, fluorescence of 4-MU with and without the presence of compounds was compared. The percentage of quenching was determined by setting the fluorescence of 4-MU to 100%. With the exception of **3** (15.41% compared to test buffer without compound), self-fluorescence of the test compounds was generally low (<4%). Quenching of FL of about 40% and 30% was determined for compounds **4** and **5**, respectively. All licorice constituents tested inhibited bacterial NA in both the CL and FL assay. In comparison to viral NA, the IC_{50} values determined for *C. perfringens* NA were about 10 times lower in the CL assay. High concentrations of the virus-specific NAI oseltamivir had to be added to inhibit this bacterial NA by 50% as reported previously.²¹

CONCLUDING REMARKS

The lack of reliable and effective anti-influenza drugs and threat of a devastating influenza pandemic have created an urgent demand for novel therapeutic options. In this regard, the impact of compounds from natural sources, as identified rather by academic than industrial groups, has proven to be substantial. The application of in silico-guided approaches for the target-oriented identification of influenza NAIs has emerged as a promising way to identify such natural leads. It should be noted that this approach will not lead to new molecules since yet undiscovered natural compounds are not entities of any library used for virtual screening. However, shape-based similarity screening, as performed in this study, allows for scaffold hopping to compounds with backbones differing from those of their templates. In this case, the templates belonging to the chemical classes of neolignans (honokiol, template A) and diarylheptanoids (katsumadain A, template B) led to the identification of structurally diverse compounds with unprecedented bioactivities.

Shape-based similarity screening of the TCM Database@Taiwan was used as a tool for the targeted selection of a plant material, namely, *G. glabra* (licorice), which produces several molecules showing similarities to previously identified NAIs. Licorice is one of the oldest and most popular traditional herbal medicines in traditional Oriental medicine and is found in

complex formulas frequently used to treat respiratory infections.

Although there are many reports on the anti-influenza activity of licorice extracts or preparations,²⁹ investigations on the target-based molecular level have been limited. Herein, the aim was to get a better understanding of the mechanism of anti-influenza action of licorice and its constituents. Of 12 isolated and identified constituents, 11 showed a distinct influenza NA inhibition in a CL-based assay. The licorice constituents were further characterized by using (i) a cell-based CPE inhibition assay (general antiviral activity), (ii) an evaluation of cytotoxicity, (iii) an inhibition assay for *C. perfringens* (CL- and FL-based assay) NA, and (iv) a determination of self-fluorescence as well as quenching. The latter test enables false-positive results to be excluded as reported particularly for flavonoid scaffolds.²¹ In addition, it was observed that good NA inhibition is not necessarily reflected by a general antiviral effect in the CPE assay. This might be due to differences in the experiment setup for assays using an isolated enzyme as opposed to cell-based assays. In cell-based assays, a suboptimal balance of hemagglutinin receptor-binding affinity and NA receptor-cleaving activity can affect the susceptibility to NAs, which hampers the interpretation of results.¹⁰

Considering possible assay interference problems, it was concluded that licorice compounds **1**, **3**, **5**, and **6** showed distinct anti-influenza activities that are most likely a result of the inhibition of NA. Of these four compounds, three (compounds **1**, **3**, and **6**) gave reasonably high scores in shape-focused virtual screening (TanimotoCombo above 0.70). When comparing the template activities to the final biological results of identified licorice compounds, similar activities were found for compounds **3** and **6** derived from templates A and B, respectively. Intriguingly, compound **1** showed a 10-fold higher in vitro activity than its corresponding template A.

Recent studies applying a systems-wide approach indicated that one quarter of biologically active licorice components are orally bioavailable.⁴⁶ In another study, the pharmacokinetic profiles of major known bioactive licorice constituents (including flavones, chalcones, isoflavones, saponins, and coumarins) were compared to the profile of a complex licorice extract. Interestingly, as opposed to the profile of the individual constituents applied, interactions of compounds from the extract indicated an improvement in bioavailability especially for aglycones.⁴⁷ Taking these aspects into account, the identified bioactive compounds, i.e., aglycones, from our study might be made more bioavailable by application as a traditionally used multicomponent mixture rather than as single individual compounds.

In conclusion, the present results show that a combination of a computational approach with an experimental evaluation at both the compound- as well as the pharmacological target-level is a powerful tool to shed light on the biological action of well-approved traditional remedies. In particular, the molecular mode of action of multicomponent and multitargeting herbal mixtures, as frequently found in traditional medicines based on holistic ideas, might be critically analyzed on a molecular level and unraveled in a straightforward way.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter at 25 °C. 1D and 2D NMR experiments were performed by using Bruker DRX300 and TXI600 NMR spectrometers, operating at 300 and 600 MHz,

respectively. The samples were measured in MeOD (calibrated to the residual nondeuterated solvent signals). MS analysis was performed on an Esquire 3000 Plus ion-trap mass spectrometer (Bruker Daltonics) equipped with electrospray ionization (ESI) in the positive and negative modes: spray voltage, 4.5 kV; sheath gas, N₂, 30 psi; dry gas, N₂, 6 L min⁻¹, 350 °C; scanning range, *m/z* 50–1000. Column chromatography was performed using Merck silica gel 60 (40–63 μm) and Pharmacia Sephadex LH-20 (20–100 μm). The fractions obtained from all chromatographic steps were analyzed by TLC (mobile phase, CH₂Cl₂–MeOH (9:1), EtOAc–EtOH–H₂O–NH₃ (13:5:1.8:0.2), or CH₂Cl₂–MeOH–HCOOH (9:1.5:0.5); stationary phase, Merck silica gel 60 PF₂₅₄, detected with staining reagents anisaldehyde/H₂SO₄ at vis, UV₂₅₄, UV₃₆₆). HPLC was performed on a Shimadzu UFLC-XR instrument (Kyoto, Japan) with a photodiode array detector. LC parameters: stationary phase, Phenomenex Luna Phenyl-Hexyl, 150 × 3.00 mm, 3 μm, Agilent; temperature, 40 °C; mobile phase, water (A); acetonitrile (B); flow rate, 0.2 mL/min; UV detection wavelength, 205, 254, 280, 310, 360 nm; injection volume, 10 μL; gradient, 80% A, 20% B, 7 min; 68% A, 32% B, 20 min; 67% A, 33% B, 23 min; 2% A, 98% B, 37 min; 2% A, 98% B. All chemicals and solvents used were analytical grade. Honokiol (98% purity) was purchased from Quality Phytochemicals LLC, East Brunswick, NJ, USA.

Plant Material. The roots of *G. glabra* L. were obtained from “Mag. Kottas – Heilkräuter”, Eitnergasse 8, 1230 Vienna, Austria (A924304-001). Voucher specimens (JR-20091106-A1; JR-20091203-E1) are deposited in the Herbarium of the Institute of Pharmacy/Pharmacognosy, LFU, Innsbruck, Austria.

Extraction and Isolation. The dried ground roots of *G. glabra* (529.6 g) were macerated with MeOH (at room temperature, three times for 72 h each). After removal of the solvent under vacuum, the MeOH extract (87.0 g) was obtained. Another 232.8 g of dried ground roots of *G. glabra* was extracted with 1 L of 1 N H₂SO₄ for 2 h at a temperature of 100 °C to hydrolyze the glycosides. The extract obtained was partitioned with CH₂Cl₂ in order to separate the aglycones from sugars in the remaining aqueous phase.

The methanol extract was partitioned between EtOAc and H₂O. The EtOAc fraction (7.2 g) was subjected to silica gel column chromatography (Merck silica gel 60 PF₂₅₄, 213 g; 65 cm × 4 cm) using a gradient system of *n*-hexane–EtOAc–MeOH to give 23 fractions (A1–23). Fractions A6–9 (374.2 mg) were combined and further separated using silica gel column chromatography (Merck silica gel 60 PF₂₅₄, 213 g; 65 cm × 4 cm) applying a gradient system of CH₂Cl₂–acetone to yield 23 fractions (B1–23). Fraction B7 (3.3 mg) was purified via Sephadex LH-20 column chromatography (mobile phase: MeOH) to give 1.3 mg of compound **10**. Fraction A13 (278.0 mg) was subjected to Sephadex LH-20 column chromatography (mobile phase: MeOH), yielding eight fractions (D1–8). From fraction D8 was obtained 24.3 mg of pure compound **8**. Fraction A14 (942.5 mg) was chromatographed over a Sephadex LH-20 column eluted with MeOH to give six fractions (E1–6). Fraction E4 (330.0 mg) was subjected to silica gel column chromatography (Merck silica gel 60 PF₂₅₄, 213 g; 65 cm × 4 cm) eluting with a gradient solvent system of CH₂Cl₂–MeOH, yielding seven fractions (F1–7). Fraction F3 (150.3 mg) was further separated by means of a Sephadex LH-20 column (mobile phase: MeOH) to give four fractions (G1–4). Fraction G1 (67.5 mg) was purified by a Lobar chromatography system (Merck Lobar commercial column Art.: 10624; size: A (240–10); LiChroprep RP-18 40–60 mm; gradient elution with CH₃CN–H₂O) to give 16.5 mg of compound **11**. The combined fractions G3–4 (41.9 mg) were purified by Lobar chromatography (gradient elution with CH₃CN–H₂O) to give 1.4 mg of compound **12**. Fraction E6 (34.1 mg) was subjected to Lobar chromatography (gradient elution with CH₃CN–H₂O), yielding six fractions (J1–6). Fraction J4 (8.2 mg) was purified via a Sephadex LH-20 column (mobile phase: MeOH), resulting in 7.1 mg of compound **9**.

The aglycone-enriched fraction (2.2 g) was submitted to passage over a Sephadex LH-20 column (mobile phase: CH₂Cl₂–acetone) to yield seven fractions (A1–7). Fractions A4–6 (1.0 g) were combined and chromatographed over a silica gel column using a gradient solvent

system of hexane–CH₂Cl₂–MeOH, resulting in the collection of 12 fractions (B1–12). Fraction B2 (8.0 mg) was separated via a Sephadex LH-20 column (mobile phase: MeOH), giving three fractions (C1–3). Fraction C1 yielded 3.7 mg of compound 6. Fraction B5 (31.3 mg) was further purified via a Sephadex LH-20 column eluted with MeOH to give five fractions (D1–5). Compound 2 (3.1 mg) and compound 1 (1.5 mg) were obtained from fractions D1 and D5, respectively. Fraction B7 (110.4 mg) was separated on a Sephadex LH-20 column eluted with MeOH to yield seven fractions, E1–7. Fraction E3 (39.9 mg) was subjected to Lobar chromatography (gradient elution with MeOH–H₂O), yielding five fractions (F1–5). Then, 23.5 mg of fraction F4 was subjected to Sephadex LH-20 column chromatography (mobile phase: MeOH), yielding six fractions (G1–6). From fraction G3 was obtained 5.6 mg of pure compound 4. Fractions E4 and E5 were combined (22.9 mg) and subjected to Lobar chromatography (gradient elution with MeOH–H₂O), yielding five fractions (H1–5). Among them, H3 afforded 2.2 mg of pure compound 5. Fraction B8 (351.4 mg) was separated via a Sephadex LH-20 column using MeOH as the eluent. This separation resulted in five fractions (I1–5), with 83.9 mg of fraction I3 further purified via Lobar chromatography (gradient elution with MeOH–H₂O), yielding a further 12 fractions (J1–12). Fraction J10 (5.7 mg) was purified via passage over a Sephadex LH-20 column (mobile phase: MeOH), yielding 2.8 mg of pure compound 7. Next, 48.3 mg of fraction I4 was purified via Lobar chromatography (gradient elution with MeOH–H₂O) to give 3.9 mg of pure compound 3.

The physical and spectroscopic data of compounds 1 to 12 agreed with those published previously for (*E*)-1-[2,4-dihydroxy-3-(3-methyl-2-butenyl)phenyl]-3-(8-hydroxy-2,2-dimethyl-2H-1-benzopyran-6-yl)-2-propen-1-one (1), 3,4-dihydro-8,8-dimethyl-2H,8H-benzo[1,2-*b*:3,4-*b'*]dipyran-3-ol (2), biochanin B (3), glabrol (4), glabrone (5), hispaglabridin B (6), licoflavone B (7), licorice glycoside B (8), licorice glycoside E (9), liquiritigenin (10), liquiritin (11), and prunin (12).^{33–41,48} Their purity was checked using TLC and LC-MS and was revealed to be >98% in all cases.

Virtual Screening. TCM Database@Taiwan version 1.0 contains about 40 000 molecules related to traditional Chinese medicines. The database was converted into MDL SD file format using Schrödinger Maestro Suite (version 9.2).⁴⁹ Conformational models for all compounds were calculated using Omega (version 2.3.2 OpenEye Scientific Software, Santa Fe, NM, USA),⁵⁰ with default settings. In total, 34 722 molecules successfully passed these preparation steps. ROCS (version 2.4.2 OpenEye Scientific Software)⁵¹ was used to rank all molecules by decreasing similarity to the templates A and B. The minimum energy conformation for each template (calculated with Omega, default settings) was used as ROCS query.

Hardware Setup. Omega and ROCS calculations were run on an Apple MacBook Air with an Intel Core i7 1.8 GHz CPU and 4 GB of RAM under Mac OS X version 10.7.

Cell Culture and Viruses. H1N1 influenza viruses A/Jena/8178/09 (J/8178/09; isolated from nasal swabs of an influenza patient during the pandemic of 2009), A/PR/8/34 (PR/8/34; Department of Virology and Antiviral Therapy, Jena, Germany), and A/Berlin/55/08 (B/55/08; oseltamivir-resistant, seasonal H1N1 isolate, published using the sample number 342/09;¹³ Robert-Koch-Institute, Berlin, Germany) as well as the H3N2 strain A/HongKong/68 (HK/68; strain collection of the Department of Virology and Antiviral Therapy, Jena, Germany) were used in antiviral studies. They were propagated in Madin Darby canine kidney (MDCK) cells (Friedrich-Loeffler Institute, Riems, Germany) in serum-free Eagle's minimum essential medium supplemented with 100 U/mL penicillin as well as streptomycin, 2 µg/mL trypsin, and 1.2 mM bicarbonate.⁵² Titers of virus stocks were determined according to Reed and Muench, 1938 in MDCK cells.⁵³

FL- and CL-Based NA Inhibition Assays. Inhibition of the NA of influenza A viruses and/or *C. perfringens* (Sigma-Aldrich, Chemie GmbH, Munich, Germany) was analyzed by applying the FL- and/or CL-based NA inhibition assays NA-Fluor Influenza Neuraminidase Assay Kit and NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (both Applied Biosystems, Darmstadt, Germany),

according to the manufacturer's instructions. For determination of 50% inhibitory concentrations (IC_{50s}), NA inhibition was tested with serial log 10 (FL assay) or half-log (CL assay) NAI concentrations, as previously published for oseltamivir and zanamivir (maximum tested concentration for pure compounds: 100 µM).¹¹ Six untreated virus controls were included. Oseltamivir carboxylate (Tamiflu; Hoffmann-La Roche AG, Basel, Switzerland) was used as a positive control. FL and CL were read using a microtiter plate luminometer (FLUOstar Omega, BMG-LABTECH GmbH, Ortenberg, Germany). At least three independent assays were performed. Means and standard deviations of IC₅₀ values were calculated by linear interpolation (PR/8/34 and B/55/08) and/or according to recommendations of the Centers of Disease Control and Prevention (CDC)¹¹ with the JASPR curve fitting software (kindly provided by Larisa V. Gubareva, CDC). Additionally, self-fluorescence and quenching of compounds were analyzed using 100 µM compound solutions. To calculate the percentage of self-fluorescence of the test compounds, the fluorescence of 4-methylumbelliferone (4-MU, the fluorescent cleavage product) in the test buffer was set to 100%. To determine quenching, the fluorescence of 3.1 µM of 4-MU without and with 100 µM of compounds was compared. To calculate the percentage of quenching, the fluorescence of 4-MU was set at 100%.

Determination of Cytotoxicity and Cytopathic Effect Inhibition. The 50% cytotoxic concentration (CC₅₀) as well as 50% inhibitory concentration (IC₅₀, inhibition of virus-induced CPE) was determined on two-day-old confluent MDCK cell monolayers grown in 96-well plates as described previously (maximum tested concentration: 50 µM).⁵⁴ Cytotoxicity was analyzed 72 h after adding the test compounds. CPE inhibition was measured 48 h after infection. A multiplicity of infection of 0.001 and 0.003 TCID₅₀/cell of J/8178/09 (pandemic H1N1) and HK/68 (H3N2) resulted in a complete CPE at this time point. Each concentration was tested in triplicate or once for determination of cytotoxicity and CPE inhibition, respectively. The mean CC₅₀ and IC₅₀ values and standard derivations were calculated from at least two (J/8178/09) or three (HK/68) experiments.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank E. P. Ellmerer (Institute of Organic Chemistry, University of Innsbruck) for NMR measurements, B. Jahn (Department of Virology and Antiviral Therapy, Jena University Hospital) for technical assistance, K. M. Getzner and M. E. Koch (Institute of Pharmacy/Pharmacognosy, Center for Molecular Biosciences Innsbruck, University of Innsbruck) for assistance in phytochemical processing, and J. E. Fuchs (Institute of General, Inorganic and Theoretical Chemistry, Center for Molecular Biosciences Innsbruck) for discussion of computational aspects. This work was supported by the Austrian Science Fund (FWF: P24587 and P23051) and the European Social Fund (ESF and TMWAT Project 2011 FGR 0137).

DEDICATION

Dedicated to Prof. Dr. Otto Sticher, of ETH-Zurich, Zurich, Switzerland, for his pioneering work in pharmacognosy and phytochemistry.

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